16S/18S rRNA PCR Library Creation

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Summary

This protocol is to (i) check the purity of DNA used for genome shotgun sequencing of single organisms and (ii) evaluate the diversity of an environmental DNA sample.

EH&S

JGI employee performing this procedure must wear a lab coat, safety glasses, and gloves at all times.

Materials & Reagents – PCR amplification and Cloning

Materials/Reagents/Equipment	Vendor	Stock Number
Disposables		
384-well PCR Microplate, Blue	Abgene	TF-0384/B
Clear Adhesive Plate Sealers	Edge BioSystems	48461
Reagents		
Milli-Q Water	Millipore Milli-Q System	-
27F primer (AGAGTTTGATCCTGGCTCAG)	IDT or Operon or your favorite	-
4aF primer (TCCGGTTGATCCTGCCRG)	IDT or Operon or your favorite	-
1391R primer (GACGGGCGGTGWGTRCA)	IDT or Operon or your favorite	-
515F (GTGCCAAGCAGCCGCGGTAA)	IDT or Operon or your favorite	-
1209R (GACGGGCRGTGWGTRCA)	IDT or Operon or your favorite	-
Escherichia coli genomic DNA	ATCC	10798D-5
Archaeoglobus fulgidis genomic DNA	ATCC	49558D
Mink genomic DNA	ATCC	CCL-64D
Easy-A High-Fidelity PCR Master Mix	Stratagene	600640
6X loading dye	See reagent/stock preparation	-
Agarose, for routine use	Sigma	A9539-250G
50X TAE Buffer	Invitrogen	24710-030
Ultra Pure Ethidium Bromide (10mg/ml)	Invitrogen	15585011

1 Kb DNA ladder	Invitrogen	15615-016
TOPO TA Cloning Kit for Sequencing	Invitrogen	K4580-40
Equipment		
CLP Agarose gel box (12cm x 14cm) and two 25 teeth combs	CLP	75.1214-MT-25D

Procedure – PCR amplification and Cloning

NOTE: *All reagents/stock solutions should be prepared prior to the start of the procedure.*

NOTE: *Cloning should be performed on the same day as PCR amplification.*

1. **16S/18S rRNA gene PCR amplification**

1.1 Add **~100 ng** of DNA template to the bottom of a clean, well-labeled 96-well plate and per primer set required.

NOTE: *There are three primer sets for rRNA gene amplification as follows:*

27F/1391R for bacterial 16S rRNA gene amplification (BAC 16S)

4aF/1391R for archaeal 16S rRNA gene amplification (ARCH 16S)

515F/1209R for eukaryotic 18S rRNA gene amplification (EUK 18S)

NOTE: *draw out which samples require amplification with which primer set and use different plates or plate sections for each primer set required.*

NOTE: *For environmental samples: leave 2 blank rows per row used!*

1.2 Add a positive control and a negative control per primer set used:

a. Step 2.a add **~10 ng** of control DNA to the bottom of the 96-well plate:

E. coli DNA for BAC 16S, *A. fulgidis* genomic DNA for ARCH 16S, mink genomic DNA for EUK 18S

b. Step 2.b add **18.9 µl** of nuclease free H_2O to the bottom of the 96-well plate.

1.3 Bring each sample volume up to **18.9 µl** with nuclease free H_2O.

1.4 Set-up the following PCR master mix for each primer combination for the amount of samples plus positive and negative controls. Keep plate on ice.

	1X	**12X**
F primer (10 µM)	1.8 µl	21.6 µl
R primer (10 µM)	1.8 µl	21.6 µl
Easy-A Master Mix	22.5 µl	270 µl
Total Volume:	**26.1 µl**	**313.2 µl**

1.5 Dispense **26.1 µl** of the PCR mix into each PCR plate well with template DNA. Keep plate on ice.

1.6 Spin down the plate.

1.7 Make a visual check to make sure all of the wells have mix in them (**45 µl**/ well).

1.8 Using the multi-channel pipettor, mix the samples by pipetting up and down gently. Be careful of your well location and of contamination.

1.9 **For environmental samples only:** using the multi-channel pipettor transfer 2 times 15 µl/ sample into the rows below.

NOTE: *You will end up with* ***3x 15 µl****/ sample.*

1.10 Quick spin plate.

1.11 Set up a PE 9700 with the following PCR program (This is a 1.5 h protocol):

94 °C – 3 min.

94 °C – 30 sec.
55 °C – 30 sec.
68 °C – 90 sec.
} **20 cycles**

72°C – 10 min.
4 °C – ∞

1.12 **For environmental samples only:** using the multi-channel pipettor pool the samples back into one **45 µl** aliquot/ sample and mix the samples by pipetting up and down gently. Be careful of your well location and of contamination.

2. 2. QC agarose gel separation

2.1 Transfer a **10 µl** aliquot per sample to a fresh PCR plate (or tubes).

2.2 Add **2 µl** of 6X loading dye per sample.

2.3 Vortex and spin quickly to collect the sample.

2.4 Load **12 µl** of sample onto 1% agarose gel containing Ethidium Bromide and save the remaining sample for cloning (on ice). Load **10 µl** of 1 Kb DNA ladder between the samples.

NOTE: *use smallest comb size for the agarose gel (i.e. 25 teeth combs for 12cm x 14cm agarose gel box).*

CAUTION! *Use proper safety precautions while handling Ethidium Bromide. This is a carcinogen/irritant.*

2.5 Run gel for ~ 40 min at 120V.

2.6 Image gel and check for proper inserts at **~1.4 kb** (BAC16S), **~1.4 kb** and/or **~2.1 kb** (ARCH16S) and **~0.7 kb** (EUK 18S). Make sure there is no non-specific amplification.

NOTE: *you should see a single discrete band (exceptions are possible; i.e. archaeal 16S rRNA genes with introns; some variations in rRNA gene size).*

If you see more than a discrete band of the expected size or smearing from your PCR, you either (i) optimize your PCR to eliminate non-specific amplification or (ii) gel-purify your fragment prior to cloning (avoid nuclease contamination when gel purifying!)

NOTE: *make sure your positive control is positive and your negative control negative.*

2.7 Discard the gel in the appropriately labeled waste.

3. **Ligation**

3.1 Add **0.5-4 µl** of fresh PCR product to the bottom of a clean, well-labeled 96-well plate (do not include negative controls).

NOTE: use PCR product volumes based on the intensity of the PCR product on the gel (i.e. use 4 µl, if very faint).

3.2 Add **1 µl** of dilute salt solution.

3.3 Bring the sample volume up to **5 µl** with nuclease free H_2O.

3.4 Add **1 µl** of TOPO vector.

3.5 Mix gently and quick spin.

3.6 Incubate for 30 min at room temperature.

Materials & Reagents – Transformation

Materials/Reagents/Equipment	**Vendor**	**Stock Number**
Disposables		
Gene Pulse Cuvette 0.1 cm electrode gap	BioRad	165-2089
Falcon 14 ml Polypropylene Tube	Becton Dickinson	352059
Cryogenic Vial	Corning	430289
LB Carb 150 X-gal Plates	Teknova	L4940

Reagents		
ElectroMAX DH10B Cells	Invitrogen	18290015
SOC Medium	Teknova	S1640
SOC Medium	Invitrogen	Supplied with comp cells
Glycerol	Sigma	G-6279
Equipment		
Gene Pulser II	BioRad	-
Pulse Controller Plus	BioRad	-

Procedure – Transformation

NOTE: *All reagents/stock solutions should be prepared prior to the start of the procedure.*

1. **Equipment Settings (BioRad Pulse Controller):**
   - Low range: 200
   - High range: ∞ (not used)
   - Capacitance: 25
   - Voltage: 1.8 kV

2. **Transformation**

   2.1 Place on ice: one well-labeled Eppendorf tube and cuvette for each sample being transformed.

   2.2 Thaw ElectroMax DH10B competent cells on ice (each tube contains 100µl, enough for 2 reactions). Discard unused cells-DO NOT refreeze!

   2.3 To the appropriately well-labeled, cold Eppendorf tube; add **1 µl** of ligation product.

   2.4 Once thawed, mix competent cells by swirling with pipette tip a few times.

   2.5 Add **50 µl** eDH10B competent cells to the Eppendorf tube.

   2.6 Mix by swirling the ligation and competent cells together with pipette tip a few times.

   2.7 Transfer solution to the bottom groove of COLD cuvette and tap on tabletop a few times to settle solution to the bottom (must see even levels of cells on each side of the cuvette without bubbles).

   2.8 Electroporate at 1.8 kV.

   2.9 Transfer cell solution immediately to **950 µl** of SOC in a 14ml falcon tube at room temperature (make sure SOC is clear, i.e. no growth).

Important! *Transfer electroporation within 10 seconds.*

2.10 Rinse cuvette with **50 µl** of the same SOC mixture to which you just added the cells.

2.11 Incubate within rotating wheel at 37°C for 1 hour.

2.12 After incubation, place on ice (no more than one hour) until ready to plate on agar plates.

3. **Plating with Beads:**

3.1 Before transforming, prepare one well-labeled LB/CARB150/IPTG/X-gal agar plate per library by letting them warm to 37˚C in an incubator to dry agar down and open (~ 90 min).

3.2 After 60 minute transformation incubation, make a 10% glycerol transformation stock (**143 µl** 80% glycerol + full transformation = ~ **1143 µl** glycerol transformation stock).

3.3 Cap, then mix transformation glycerol stock by inverting several times. [Can store tube at -80˚C if not plating within 10 min.]

3.4 Prepare a tube with **1.5 ml** of SOC (plating tube).

3.5 Add **10 µl** of the transformation glycerol stock to 1.5ml of SOC (plating tube).

3.6 Transfer remaining transformation glycerol stock into cryotube and store immediately at –80˚C for long term storage.

3.7 Mix tube by inverting several times (plating tube).

3.8 Place 10-15 beads onto bioassay.

3.9 Pipet entire volume of plating tube onto bioassay with beads (~**1510 µl**). Pipet stock all over plate.

3.10 Shake beads around on bioassay until the stock has been evenly spread (Be Gentle!).

3.11 Place bioassay on benchtop to dry completely.

3.12 When bioassay is dry, pour beads off of bioassay into autoclave waste.

3.13 Incubate the bioassays agar down in 37˚C incubator for 16-18 hrs.

Materials & Reagents – QC PCR

Materials/Reagents/Equipment	**Vendor**	**Stock Number**
Disposables		
96-well PCR plate	USA Scientific	1402-9708
Reagents		
Taq DNA Polymerase	Amersham Biosciences	27-0799-63

10X PCR Buffer	Amersham Biosciences	-
10mM dNTP mix	MBI Fermentas	R0192
pUC-F primer (CTTTACACTTTATGCTTCC)	IDT or Operon or your favorite	-
pUC-R primer (GCAAGGCGATTAAGTTGG)	IDT or Operon or your favorite	-
1 Kb DNA ladder	Invitrogen	15615-016
Equipment		
GeneAmp PCR System 9700	Perkin Elmer (Applied Biosystems)	-

Procedure – QC PCR

NOTE: *All reagents/stock solutions should be prepared prior to the start of the procedure.*

1. rRNA Gene library PCR QC

1.13 Make up the following PCR master mix for each sample (i.e. 260X for 240 clones). Keep plate on ice.

	1X	**260X**
Nuclease free H_2O	17 µl	4420 µl
10X PCR Buffer	2 µl	480 µl
10mM dNTP (MBI)	0.4 µl	96 µl
pUC-F primer (10 pmol/µl)	0.2 µl	48 µl
pUC-R primer (10 pmol/µl)	0.2 µl	48 µl
Taq (Amersham)	0.2 µl	48 µl
Total Volume:	**20 µl**	**4800 µl**

1.14 Dispense **20 µl** of the PCR mix into each well needed of the PCR plate. Keep plate on ice.

1.15 Spin down the plate.

1.16 Make a visual check to make sure all of the wells have mix in them.

1.17 Using pipette tips only, pick the desired number of colonies per library (usually 12 colonies per library) into their own well. Be careful of your well location and of contamination. Mix tips in cocktail well.

1.18 Quick spin plate.

1.19 Set up a PE 9700 with the following colony PCR program (This is a ~3 h protocol):

94 °C – 4 min.

94 °C – 30 sec.
55 °C – 30 sec.
68 °C – 2 min.
} **35 cycles**

4 °C – Hold

1.20 Add **4 µl** of 6X loading dye to PCR plate.

1.21 Vortex and spin down plate to collect.

1.22 Load **12 µl** of sample onto 1% agarose gel containing Ethidium Bromide and save the remaining sample for later use if gel fails. Leave a well empty between libraries for loading **10 µl** of 1 Kb DNA ladder.

CAUTION! *Use proper safety precautions while handling Ethidium Bromide. This is a carcinogen/irritant.*

1.23 Run for ~ 30 min at 120V.

1.24 Image gel and check for inserts ~1.55 kb (BAC 16S), ~1.6 and/or ~2.3 (ARCH 16S), 0.9 kb (EUK 18S).

1.25 Discard the gel in the appropriately labeled waste.

Reagent/Stock Preparation

80% Glycerol Stock Solution

40ul 100% Glycerol (pipette slowly)
10ul Nuclease-free H_20
Autoclave
Store at room temperature

6X Loading Dye
75ml 100% glycerol
125ml Nuclease free H_20
0.05g Bromphenol Blue
0.05g Xylene Cyanole FF
Store at 4˚C

1X TAE Buffer

To make 20 liters:
400ml 50X TAE Buffer
19.6L Milli-Q H_2O
Store at room temperature

Troubleshooting

Section on PCR optimization and/ or gel extraction for samples lacking specific amplification.

SOP Approval

DEPARTMENT	APPROVED BY	DATE
Lab Supervisor		
Research & Development		
Instrumentation		
QC		
Purchasing		
EH & S		
Informatics		
Seq Assessment & Analysis		
Dept Head of Prod Seq		

Appendix

AUDIT TRACKING

PROCEDURAL CHANGES